Molecular Subtyping of Human T-Lymphotropic Virus Type I (HTLV-I) by a Nested Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis of the Envelope Gene: Two Distinct Lineages of HTLV-I in Taiwan

Ya-Chien Yang[1,2], Tsuey-Ying Hsu[1], Mei-Ying Liu[1], Ming-Tseh Lin[3], Jen-Yang Chen[1], and Czau-Siung Yang[1]*

[1]*Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taiwan, Republic of China*
[2]*School of Medical Technology, College of Medicine, National Taiwan University, Taiwan, Republic of China*
[3]*Department of Oncology, National Taiwan University Hospital, Taipei, Taiwan, Republic of China*

The major type of human T-lymphotropic virus type I (HTLV-I), generally referred to as the cosmopolitan type, has been grouped into three subtypes (A, B, and C) by phylogenetic analysis of the long terminal repeat sequences of the viral genome. Twelve subtype-specific nucleotide variations have been deduced by comparison between the envelope (*env*) sequences of 16 HTLV-I strains with defined subtypes and 9 Taiwanese HTLV-I strains. To gain further insights into the molecular epidemiology of HTLV-I and the origin of this virus in Taiwan, a rapid method of identification for the cosmopolitan subtypes was developed by using a nested polymerase chain reaction (PCR) and subsequent restriction fragment length polymorphism (RFLP) studies using the two subtype B-specific and four subtype C-specific nucleotides located within the positions 5708 to 6320 of the *env* gene. The nested PCR-RFLP method was used to subtype HTLV-I from four virus-positive cell lines derived from 1 Japanese and 3 North American patients, as well as 41 blood-unrelated Taiwan Chinese. The sequences of PCR products were determined and the six positions of subtype-specific nucleotide variations were examined. The sequence data completely supported the subtyping data via the nested PCR-RFLP method. The results demonstrated that, as is the case in Japan, at least two distinct cosmopolitan subtypes (A and B) of HTLV-I were present in Taiwan, but the more prevalent subtype in Taiwan is A in contrast to subtype B in Japan. Furthermore, rapid subtyping could facilitate molecular epidemiological studies of HTLV-I infection and linkage between HTLV-I subtypes and virus-associated diseases. ***J Med Virol 51:25–31, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: HTLV-I subtypes; *env* gene; subtype-specific nucleotides

INTRODUCTION

Human T-lymphotropic virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia/lymphoma (ATL) [Poiesz et al., 1980; Yoshida et al., 1982] and is closely associated with a neurological disorder named HTLV-I-associated myelopathy/tropic spastic paraparesis (HAM/TSP) [Gessain et al., 1985; Osame et al., 1986]. The virus has also been implicated in other chronic inflammatory disorders, including some cases of polyarthritis [Nishioka et al., 1989], polymyositis [Evans et al., 1989], Sjogren's syndrome [Green et al., 1989], infectious dermatitis [LaGrenade et al., 1990], and uveitis [Mochizuki et al., 1992], but the complete spectrum of diseases induced by HTLV-I is not fully defined. HTLV-I is endemic in southwestern Japan, the Caribbean basin, tropical Africa, Central and South America, and some regions of Melanesia [Hinuma et al., 1982; De The et al., 1985; Manns and Blattner, 1991; Yanagihara et al., 1991].

According to phylogenetic analysis of the long terminal repeat (LTR) sequences of the virus, three types of HTLV-I have been proposed: 1) the Melanesian type from remote Melanesians in Papua New Guinea and the Solomon Islands in the South Pacific Ocean and from Australian aboriginals [Gessain et al., 1991; Bas-

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*Correspondence to: Dr. Czau-Siung Yang, Graduate Institute of Microbiology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan, 100, R.O.C.

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tian et al., 1993], 2) the Zairian type isolated from central Africa [Ratner et al., 1991], and 3) the so-called cosmopolitan type isolated from the remaining worldwide endemic areas [Poiesz et al., 1980; Hinuma et al., 1981; Blattner et al., 1982; Biggar et al., 1984; Paine et al., 1991]. The cosmopolitan type has been further grouped into three subtypes: A, B, and C [Miura et al., 1994] which correspond to subtypes II, III, and I by Komurian-Pradel et al. 1992] and also to the cosmopolitan, Japanese, and West African subtypes by Ureta Vidal et al. [1994b]. Subtype A is identified in the Caribbean basin, South America, Japan, and India; subtype B is also identified in Japan and India; whereas subtype C is found in the Caribbean basin and West Africa.

Taiwan is located between Japan and thee southwestern Pacific Islands. Historically, a part of Taiwan had been occupied by the Spanish and subsequently the Dutch in the 17th century and by the Japanese in the early 20th century. Ethnically, most of the residents in Taiwan are descendants of Chinese immigrants who migrated from southeast China three to four centuries ago. A seroepidemiological survey of HTLV-I in Taiwan has shown that the prevalence is 4.8 per 1,000 population [Wang et al., 1988]. Although Taiwan is a nonendemic region, patients with ATL or HAM/TSP have been sporadically encountered [Su et al., 1985; Yang et al., 1993]. On the basis of the geographic distribution and a complex historical background, it is of interest to elucidate the molecular epidemiology of HTLV-I in Taiwan, thus providing further insights into the origin of this virus on the island. Twelve subtype-specific nucleotide variations have been deduced by the comparison between the envelope (*env*) sequences of 16 HTLV-I strains with defined subtypes and 9 Taiwanese HTLV-I strains [Lin et al., 1996]. In this report, we demonstrate the coexistence of the cosmopolitan subtypes A and B of HTLV-I in Taiwan by using nested polymerase chain reaction (PCR) and subsequent restriction fragment length polymorphism (RFLP) studies. Moreover, the applicability of a nested PCR-RFLP analysis of *env* gene to epidemiological study of HTLV-I is described.

MATERIALS AND METHODS

Source and Preparation of DNA Samples

Peripheral blood mononuclear cells (PBMC) were separated by using Ficoll-Hypaque centrifugation from 41 blood-unrelated Taiwan Chinese who were HTLV-I positive as determined by particle agglutination testing (Serodia-ATLA, Fujirebio) and Western blot analysis (Cambridge Biotech) as well as by PCR [Yang et al., 1991]. Thirty of these samples were collected in the National Taiwan University Hospital, in which 10 were from patients with ATL, 10 with other hematological diseases, 8 with open heart surgery, and 1 each with HAM and liver cirrhosis. The other 11 samples were obtained from three prostitutes, four intravenous drug abusers (IVDA), and four asymptomatic carriers (Table I). PBMC from 12 healthy donors who tested negative for HTLV-I infection by all the above assays were used as negative controls. The HTLV-I-positive cell lines used were MT-2, which was derived from a Japanese patient, as well as HUT102, MJ, and $C_{91/PL}$ derived from three North American patients. The HTLV-II-positive cell line used was C344/Mo derived from a North American patient. The cells were suspended in lysis buffer which contained 0.05% SDS, 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 2 mM EDTA, and 0.05 mg/ml proteinase K. After incubation at 37°C for 4 hr, DNA was extracted by the phenol extraction method.

TABLE I. HTLV-I Subtypes Based on $env^{5708\rightarrow6320}$ Region in 41 Taiwan Chinese and in 4 HTLV-I-Positive Cell Lines

Sample	Situation[a]	HTLV-I subtype	
		Nested PCR-RFLP	Sequence[b]
WSC	ATL	A	A
BTL	ATL	A	A
JPL	ATL	A	A
SJL	ATL	A	A
TSS	ATL	A	A
JMC	ATL	A	A
LSC	ATL	B	B
BSD	ATL	B	B
KNS	ATL	B	B
THY	ATL	B	B
BRS	Hematol.	A	A
SIL	Hematol.	A	A
CMT	Hematol.	A	A
SU603	Hematol.	A	A
FIT	Hematol.	B	B
MLS	Hematol.	B	B
GHC	Hematol.	B	B
JTH	Hematol.	B	B
RMS	Hematol.	B	B
CSL	Hematol.	B	B
LMW	HAM	A	A
IJW	Open heart	A	A
TST	Open heart	A	A
PH	Open heart	A	A
MSH	Open heart	A	A
CFK	Open heart	A	A
CL	Open heart	A[c]	A
CJC	Open heart	A[c]	A
MS	Open heart	B	B
HH	Cirrhosis	A	A
IMJ	Prostitute	A	A
IJL	Prostitute	A	A
CML	Prostitute	A	A
YL197	IVDA	A	A
YL755	IVDA	A	A
YL1112	IVDA	A	A
YL1003	IVDA	B	ND
LCC	Asymp.	A	A
ML	Asymp.	A	A
ISI	Asymp.	A	A
JMCC	Asymp.	A	A
HTLV-I-positive cell lines			
MT-2		A	A
HUT102		A	A
MJ		A	A
C91/PL		A	A

[a]ATL = adult T-cell leukemia/lymphoma; Hematol. = hematological diseases other than ATL; HAM = HTLV-I-associated myelopathy; IVDA = intravenous drug abuser; Asymp. = asymptomatic.
[b]Subtypes according to the sequence data at the six positions of subtype-specific nucleotides; ND = not done.
[c]Loss of *Hpa*I site at position 5846 resulting from a G-to-A mutation at position 5846.

Nested PCR Amplification

A 657 base pair (bp) fragment of the *env* gene (positions 5684–6340 according to the ATK-1 sequence in EMBL data base) was amplified by nested PCR with C (sense, positions 5642-5665, 5′-CCTCAATATTAATCTCCATTTTTC-3′) plus D (antisense, positions 6360-6341, 5′-AGAACAGGAGATCAAGGCCT-3′) as external primers and A (sense, positions 5684-5707, 5′-CTCCCTTCTAGTCGACGCTCCAGG-3′) plus B (antisense, positions 6340-6321, 5′-CGTCTGTTCTGGGCAGCATA-3′) as internal primers. All amplifications were conducted in a total volume of 100 μl which contained 200 μM each of dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10 mM Tris-HCl (pH 8.8), 300 nM each of primers, 2.5 units of DyNAZyme II DNA polymerase (Finnzymes Oy), and template DNA overlaid with 50 μl of mineral oil. Amplification reactions were performed in an automatic thermal cycler (Perkin-Elmer-Cetus) for 40 cycles of amplification (96°C for 30 sec, 58°C for 15 sec, and 74°C for 1 min) followed by a final cycle of extension (74°C for 7 min).

RFLP Analysis

According to the two subtype B-specific and four subtype C-specific nucleotide variations within the $env^{5708\rightarrow6320}$ region (positions 5775, 5795, 5837, 5903, 5949, and 6269) [Lin et al., 1996], endonucleases *Hpa*I and *Hinf*I were selected to perform RFLP analysis because of the following considerations: 1) in addition to the modified restriction site, endonucleases with a universal cleavage site in three cosmopolitan subtypes are preferentially selected to monitor the effective enzyme reactions (Fig. 1); 2) the digested DNA fragments are distinguished easily on agarose gel. As shown in Figure 1, digestion of the PCR products with *Hpa*I results in 165 and 492 (423 + 69) bp fragments in HTLV-I subtypes A and C, but 165, 423, and 69 bp fragments in subtype B due to A^{6269}-to-G mutation creating a unique *Hpa*I site at position 6269. On the other hand, digestion of the PCR products with *Hinf*I results in 358 (151 + 207) and 299 bp fragments in HTLV-I subtypes A and B, and 151, 207, and 299 bp fragments in subtype C due to C^{5837}-to-T mutation creating a unique *Hinf*I site at position 5834. One tenth of the PCR product was digested with 10 units of *Hpa*I or *Hinf*I (Promega) in a total volume of 20 μl under the conditions recommended by the manufacturer. The digested DNA was electrophoresed on a 3% NuSieve-1% SeaKem (FMC Bioproducts) agarose gel. The resulting DNA bands were visualized after staining with ethidium bromide.

DNA Sequencing of PCR Products

For each sample, five independent PCR products were pooled and concentrated for gel purification and double-stranded sequence was done to discriminate in vivo mutations and base substitutions due to misincorporation during in vitro amplification. DNA products of approximately 657 bp were separated by electrophoresis on a 2% SeaKem agarose gel and purified from the gel with a JETsorb gel extraction kit (GENOMED) as recommended by the manufacturer. The purified DNA fragments were sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer-Cetus) and an ABI 373A automatic sequencer (Perkin-Elmer-Cetus).

Sequence Analysis

Nucleotide sequences were aligned and compared with those from previously published HTLV-I strains, including CH, TSP-1, MT-2, SP (subtype A), ATK-1, H5, YS (subtype B), HS35, 1010, SIE, AKR (subtype C), Mel5, MSHR-1 (Melanesian type), and EL (Zairian type) [Seiki et al., 1983; Tsujimoto et al., 1988; Malik et al., 1988; Evangelista et al., 1990; Gray et al., 1990; Komurian et al., 1991; Paine et al., 1991; Bastian et al., 1993; Gessain et al., 1993; Chou et al., 1995]. Sequence alignments were facilitated by using the software available on the Genetics Computer Group sequence analysis package, version 8.1.

Nucleotide Sequence Accession Numbers

The Genbank accession numbers of Taiwanese HTLV-I *env* gene sequences corresponding to positions 5708-6320 of ATK-1 are as following: BRS (U60903), BSD (U60904), BTL (U60905), CFK (U60906), CJC (U60907), CL (U60908), CML (U60909), CMT (U60910), CSL (U60911), FIT (U60912), GHC (U60913), HH (U60914), IJL (U60915), IJW (U60916), IMJ (U60917), ISI (U60918), JMC (U60919), JMCC (U60920), JPL (U60921), JTH (U60922), KNS (U60923), LCC (U60924), LMW (U60925), LSC (U60926), ML (U60927), MLS (U60928), MS (U60929), MSH (U60930), PH (U60931), RMS (U60932), SIL (U60933), SJL (U60934), SU603 (U60935), THY (U60936), TSS (U60937), TST (U60938), WSC (U60939), YL1112 (U60940), YL197 (U60941), YL755 (U60942).

RESULTS

All of the 41 HTLV-I-positive Taiwanese samples and 4 HTLV-I-positive cell lines could be amplified via the nested PCR using the sequential primer pairs, C/D and A/B. No appropriate-sized PCR products were amplified in 12 seronegative samples and HTLV-II-positive cells, C344/Mo. The amplified DNAs were analyzed for the restriction sites of the endonucleases *Hpa*I and *Hinf*I by the RFLP method. The RFLP patterns of the cosmopolitan HTLV-I subtypes A and B are shown in Figure 2. Of the 41 Taiwanese HTLV-1 strains, 4 of 10 from patients with ATL, 6 of 10 from patients with other hematological diseases, 1 of 8 from patients with open heart surgery, and 1 of 4 from IVDA were subtype B while the others (29 of 41: 70.7%) were subtype A (Table I). All of the four HTLV-I strains from HTLV-I-positive cell lines, MT-2, HUT102, MJ, and $C_{91/PL}$ were subtype A.

The nucleotide sequences of 44 of 45 *env*-amplified products flanked by primers A/B were determined and the six positions of subtype-specific nucleotide variations within $env^{5708\rightarrow6320}$ region were examined for subtyping. The sequence data supported completely the subtyping by the nested PCR-RFLP method (Table I). The

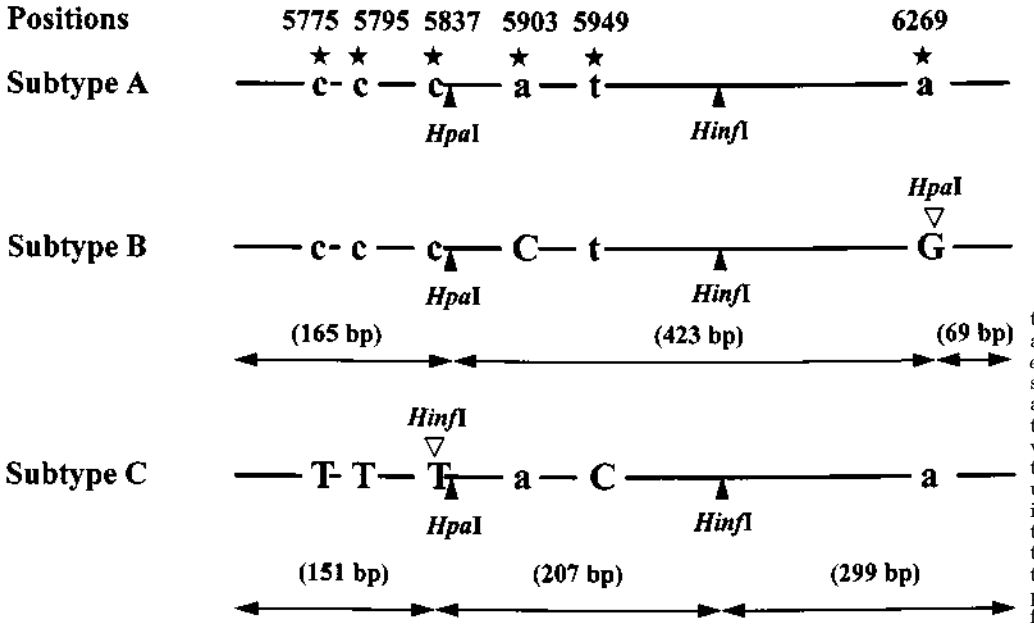


Fig. 1. *Hpa*I/*Hinf*I restriction site polymorphisms of amplified DNA in the $env^{5684\rightarrow6340}$ region. Restriction sites of endonucleases *Hpa*I and *Hinf*I are indicated. Solid triangles represent the universal cleavage sites. Open triangles represent the unique cleavage sites. Stars indicate the position of subtype-specific nucleotide variation. Numbers within parentheses indicate the size (base pairs) of the digested DNA fragments.

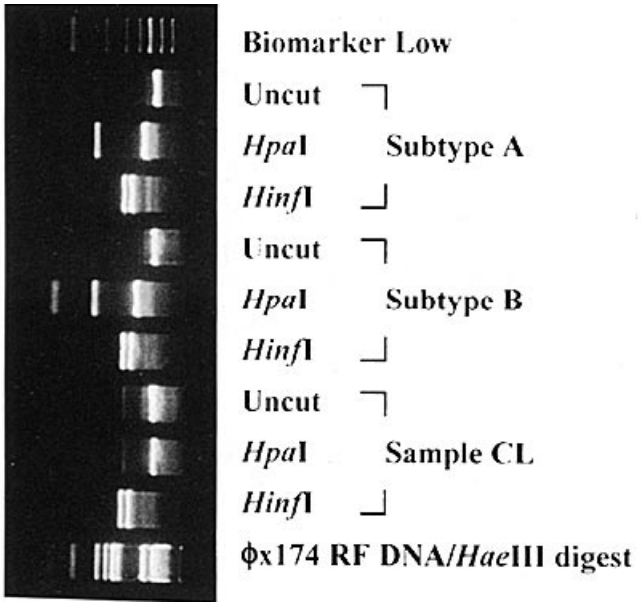


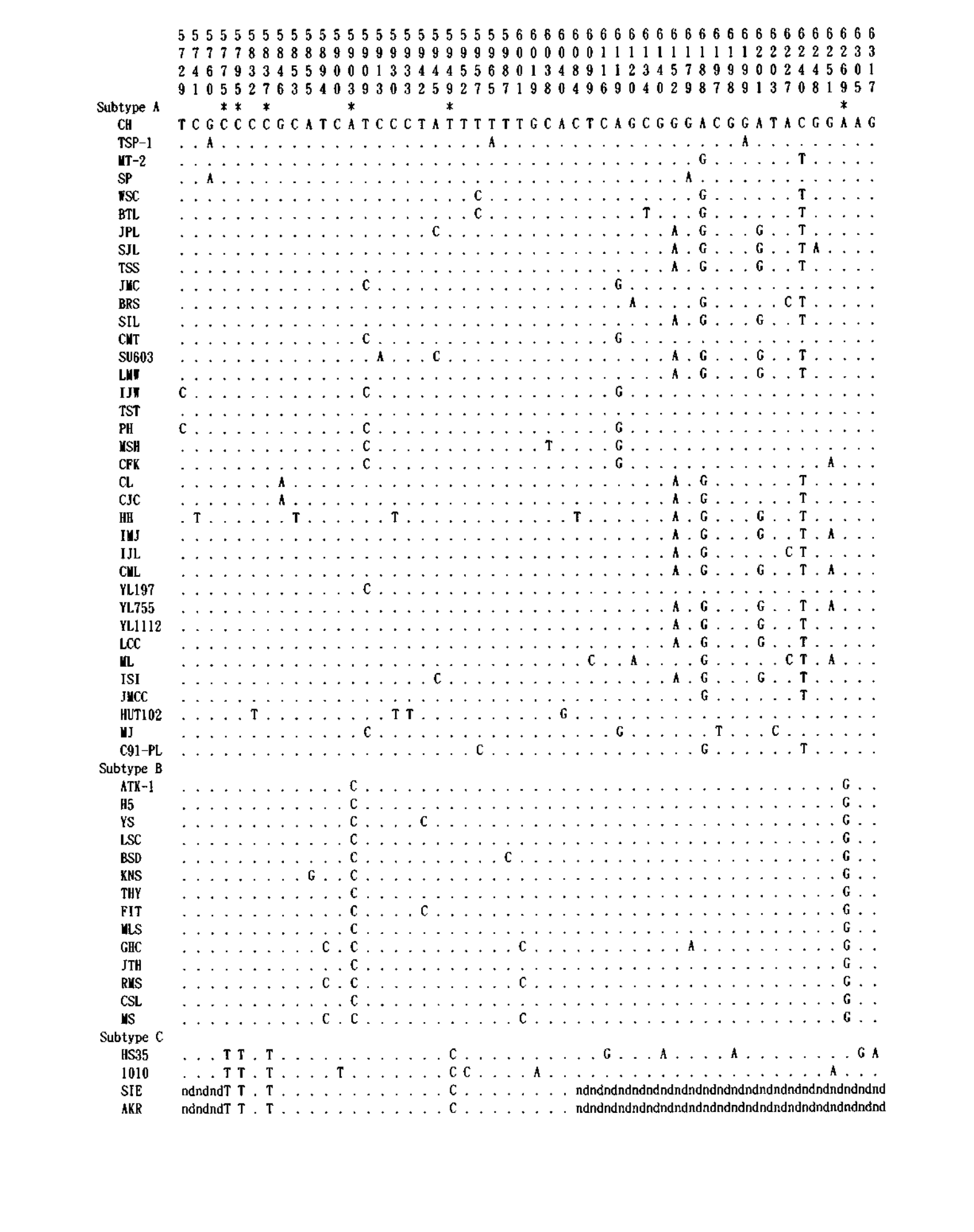
Fig. 2. RFLP analysis of *env*-amplified DNA of HTLV-I subtypes A and B using endonucleases *Hpa*I and *Hinf*I. *Hpa*I gives a universal cleavage in both subtypes and an additional unique cleavage in subtype B. *Hinf*I gives a universal cleavage in both subtypes. The universal *Hpa*I cutting site is absent in the amplified DNA of sample CL. Biomarker Low and ϕx174 RF DNA/*Hae*III digest represent DNA size markers.

alignment of the nucleotide sequences with variations within the 613 base $env^{5708\rightarrow6320}$ region of 11 HTLV-I strains with defined subtypes, CH, TSP-1, MT-2, SP (subtype A), ATK-1, H5, YS (subtype B), HS35, 1010, SIE, and AKR (subtype C), as well as those strains sequenced herein are shown in Figure 3. Sequence variations were seen at a total of 50 positions over the 613 bases sequenced. A total of 25 nucleotide substitutions were found in single individuals only, whereas 25 were shared in isolates from several individuals, in whom $G^{6179}\rightarrow A$ was found in subtypes A and B and $G^{6251}\rightarrow A$ was found in subtypes A and C. Noticeably, 4 nucleotide substitutions, $G^{6152}\rightarrow A$, $A^{6201}\rightarrow G$, $A^{6188}\rightarrow G$, and $C^{6240}\rightarrow T$, were found frequently in Taiwanese subtype A strains, in which the latter 2 were also found in the HTLV-I strains from MT-2 derived from a Japanese patient and $C_{91/PL}$ derived from a North American patient. Each amplified DNA sample contained the universal *Hpa*I cutting site at position 5846 and the *Hinf*I cutting site at position 6041, except for the CL and CJC strains, in which a G-to-A mutation at position 5846 led to the loss of the *Hpa*I cleavage at position 5846 (Figs. 2, 3). Due to lack of *Hpa*I site at position 6269, they were determined as subtype A which corresponded to the sequence data. These data clearly demonstrate that at least two distinct lineages (subtypes A and B) of HTLV-I are present in Taiwan and both subtypes were identified in patients with ATL. The majority (70.7%) of HTLV-I-positive Taiwan Chinese in this study were infected with subtype A.

DISCUSSION

To apply a rapid nested PCR-RFLP method to subtype the cosmopolitan type of HTLV-I, we have focused on the $env^{5642\rightarrow6360}$ region, which encodes the c-terminal half of gp46 and part of gp21, for the following reasons. First, the flanking primer sequences are preserved

Fig. 3. Nucleotide variations in the 613 base $env^{5708\rightarrow6320}$ region from various HTLV-I strains. The nucleotide sequences of CH strain are used for reference. Only nucleotides that differ from those in CH strain are shown and "nd" indicates that sequences are not available. On top, positions are numbered according to the scheme of Seiki et al. [1983]. Asterisks under the numbers indicate the positions of the subtype-specific nucleotide variations.


5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6
7 7 7 7 7 8 8 8 8 8 9 9 9 9 9 9 9 9 9 9 9 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 3 3
2 4 6 7 9 3 3 4 5 5 9 0 0 0 1 3 3 4 4 4 5 5 6 8 0 1 3 4 8 9 1 1 2 3 4 5 7 8 9 9 9 0 0 2 4 4 5 6 0 1
9 1 0 5 5 2 7 6 3 5 4 0 3 9 3 0 3 2 5 9 2 7 5 7 1 9 8 0 4 9 6 9 0 4 0 2 9 8 7 8 9 1 3 7 0 8 1 9 5 7
Subtype A
CH T C G C C C C G C A T C A T C C C C T A T T T T T T G C A C T C A G C G G G A C G G A T A C G G A A G
TSP-1 . . A . . . . . A . . . . . G . . . . . T . . . . .
MT-2 . . . . . G . . . . . T . . . . .
SP . . A . . . . . A . . . . . .
WSC . . . . . C . . . . . G . . . . . T . . . . .
BTL . . . . . C . . . . . T . . . G . . . . . T . . . . .
JPL . . . . . C . . . . . A . G . . . G . . T . . . . .
SJL . . . . . A . G . . . G . . T A . . . .
TSS . . . . . A . G . . . G . . T . . . . .
JMC . . . . . C . . . . . G . . . . . .
BRS . . . . . A . . . . G . . . . . C T . . . . .
SIL . . . . . A . G . . . G . . T . . . . .
CMT . . . . . C . . . . . G . . . . . .
SU603 . . . . . A . . . C . . . . . A . G . . . G . . T . . . . .
LMW . . . . . A . G . . . G . . T . . . . .
IJW C . . . . . C . . . . . G . . . . . .
TST . . . . . .
PH C . . . . . C . . . . . G . . . . . .
MSH . . . . . C . . . . . T . . . G . . . . . .
CFK . . . . . C . . . . . G . . . . . A . . .
CL . . . . . A . . . . . A . G . . . . . T . . . . .
CJC . . . . . A . . . . . A . G . . . . . T . . . . .
HH . T . . . . . T . . . . . T . . . . . T . . . . . A . G . . . G . . T . . . . .
IMJ . . . . . A . G . . . G . . T . A . . .
IJL . . . . . A . G . . . . . C T . . . . .
CML . . . . . A . G . . . G . . T . A . . .
YL197 . . . . . C . . . . . .
YL755 . . . . . A . G . . . G . . T . A . . .
YL1112 . . . . . A . G . . . G . . T . . . . .
LCC . . . . . A . G . . . G . . T . . . . .
ML . . . . . C . . A . . . G . . . . . C T . A . . .
ISI . . . . . C . . . . . A . G . . . G . . T . . . . .
JMCC . . . . . G . . . . . T . . . . .
HUT102 . . . . . T . . . . . T T . . . . . G . . . . . .
MJ . . . . . C . . . . . G . . . . . T . . . C . . . . . .
C91-PL . . . . . C . . . . . G . . . . . T . . . . .
Subtype B
ATK-1 . . . . . C . . . . . G . .
H5 . . . . . C . . . . . G . .
YS . . . . . C . . . . C . . . . . G . .
LSC . . . . . C . . . . . G . .
BSD . . . . . C . . . . . C . . . . . G . .
KNS . . . . . G . . C . . . . . G . .
THY . . . . . C . . . . . G . .
FIT . . . . . C . . . . C . . . . . G . .
MLS . . . . . C . . . . . G . .
GHC . . . . . C . C . . . . . C . . . . . A . . . . . G . .
JTH . . . . . C . . . . . G . .
RMS . . . . . C . C . . . . . C . . . . . G . .
CSL . . . . . C . . . . . G . .
MS . . . . . C . C . . . . . C . . . . . G . .
Subtype C
HS35 . . . T T . T . . . . . C . . . . . G . . . A . . . . A . . . . . G A
1010 . . . T T . T . . . . T . . . . . C C . . . . A . . . . . A . . .
SIE ndndndT T . T . . . . . C . . . . . ndndndndndndndndndndndndndndndndndndndndndndndndnd
AKR ndndndT T . T . . . . . C . . . . . ndndndndndndndndndndndndndndndndndndndndndndndndnd

throughout the HTLV-I strains with defined subtypes of which *env* sequences are available; second, the neutralizing epitopes on the envelope gp46 antigen have been located within amino acids 186 to 199 [Ralston et al., 1989; Tanaka et al., 1991] which are coded by the $env^{5708\rightarrow6320}$ region. It is worthwhile to note that a $C^{5775}\rightarrow T$ mutation in subtype C results in a $Pro^{192}\rightarrow Ser$ coding change. Therefore, it is valid to subtype HTLV-I within the $env^{5708\rightarrow6320}$ region and the issue of cross-protection among three subtypes must be dealt with before an HTLV-I vaccine can be developed.

In the early part of this century, Taiwan was occupied by the Japanese for 50 years during which time there was considerable migration and sociocultural interchange between the two regions. The cosmopolitan subtypes A and B of HTLV-I have been identified in Japan and India [Miura et al., 1994]. In the present study, the coexistence of the same subtypes was demonstrated in Taiwan. This may suggest, as proposed by Miura et al. [1994], that the Mongoloid people moving from north Asia carried these two lineages of HTLV-I into these areas or that the virus was introduced into Taiwan by the Japanese during their period of occupation and subsequently through sociocultural interchange. However, our study indicates that the more prevalent subtype in Taiwan is A in contrast to subtype B in Japan [Ureta Vidal et al., 1994a]. Additionally, according to our previous seroepidemiological study of HTLV-I in Taiwan, aboriginals have higher seropositive rates than other Taiwan Chinese [Wang et al., 1988]. Simultaneously, due to the close geographical location of Taiwan, the Malay Peninsula, and the Indonesian archipelago, it is interesting to analyze the types or subtypes as well as the nucleotide sequences of HTLV-I from aboriginals in Taiwan.

The pathogenesis of HTLV-I may be influenced by host immunological or genetic determinants [Usuku et al., 1988]. Alternatively, virus-specific factors could be, at least in part, responsible for differences in the clinical sequelae. It has been reported that neither a specific subtype of HTLV-I nor any specific mutation in the nucleotide sequences can be identified with ATL, HAM/TSP, and HTLV-I uveitis [Daenke et al., 1990; Komurian et al., 1991; Miura et al., 1994; Ono et al., 1994]. However, recent data suggest that several nucleotide changes in the U3 region of the LTR appear to be common among certain HAM/TSP isolates [Mukhopadhyaya and Sadaie, 1993] and that HTLV-I-infected individuals carrying isolates with a $T^{7959}\rightarrow C$ nucleotide mutation in the *tax* gene are implicated at higher risk for developing HAM/TSP [Renjifo et al., 1995]. Furthermore, the complete spectrum of diseases induced by HTLV-I is not fully defined and more HTLV-I-associated disorders may emerge. Therefore, it is valid to test for disease specificity among the phylogenetic subtypes and to elucidate a relationship between nucleotide sequence variations of HTLV-I and their pathogenicity. Nevertheless, when a nucleotide variation is claimed to be disease associated, caution has to be exercised to exclude its potential as a subtype-specific nucleotide. The rapid and very simple method of nested PCR-RFLP-based subtyping described here could facilitate molecular epidemiological studies of HTLV-I infection and permit large scale clinical studies of linkage between these HTLV-I subtypes and virus-associated diseases.

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